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Docket No.: 2288-0103P
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Ya-Gang XIE

Application No.: 10/735,600 Confirmation No.: 002498

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For: METHOD FOR THE DETECTION OF RISK FACTORS ASSOCIATED WITH MYOCARDIAL INFARCTION

Examiner: J. S. S. Wong

CLAIM FOR PRIORITY AND SUBMISSION OF DOCUMENTS

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Sir:

Applicant hereby claims priority under 35 U.S.C. 119 based on the following prior foreign application filed in the following foreign country on the date indicated:

Country	Application No.	Date
Canada	2414,301	December 13, 2002

In support of this claim, a certified copy of the said original foreign application is filed herewith.

Dated: April 24, 2006

Respectfully submitted,

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Canadian Patent Office

Certification

This is to certify that the documents attached hereto and identified below are true copies of the documents on file in the Patent Office.

Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,414,301**, on December 13, 2002, by **GENESIS GROUP INC.,** assignee of Ya-Gang Xie, for "Method for the Detection of Risk Factors Associated with Myocardial Infraction".

Agent certificateur/Certifying Officer

June 14, 2004

Date

Canada

(CIPO 68)
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OPIC CIPO

ABTRACT

A method for determining whether an individual is at an increased risk for myocardial infarction, comprising screening for the presence of Factor II and Factor XIII alleles associated with myocardial infarction. Also provided are kits and primers that specifically hybridize adjacent to the allele-specific regions of the Factor II and Factor XIII genes.

METHOD FOR THE DETECTION OF RISK FACTORS ASSOCIATED WITH MYOCARDIAL INFARCTION.

FIELD OF THE INVENTION

The present invention relates to the field of haemostasis, and in particular to the aspect of myocardial infarction (myocardial infarction). More particularly, the invention is directed at a method for the screening and diagnosis of myocardial infarction, especially hereditary myocardial infarction. The method according to the invention can then be used for determining the risk for myocardial infarction in individuals.

BACKGROUND OF THE INVENTION

Myocardial infarction is now among the most frequent causes of illness and death, especially in the industrial countries. If myocardial infarction is survived, the vitality of the patient is limited in most cases, by secondary symptoms such as paralysis or organ damage. There are also labor-intensive and cost-intensive follow-up treatments, such as convalescence, physiotherapy and medication to improve the health situation and prevent further complications.

Great advances have been made in recent years, especially in research into the causes of myocardial infarction, and these include cardiac tissue necrosis caused by an inadequate blood supply due to the occlusion of arterial blood vessels either by cholesterol plaque build-up, or by clot blockage (thrombosis). Risk factors for thrombosis-induced myocardial infarction are thought to include both hereditary and acquired conditions. Generally, a tendency towards myocardial infarction could arise from hyperactive coagulation pathways, hypoactive anticoagulant mechanisms, or hypoactive fibrinolysis. Mutations in genes that encode proteins in these pathways are thought to play an important role in the predisposition to myocardial infarction.

The serine protease thrombin formed by cleavage of human prothrombin(also known as Factor II), exerts a central action to the processes of thrombosis and haemostasis. The thrombin molecule plays a role in the final stage of blood coagulation: the formation of an insoluble fibrin clot.

The hitherto known congenital disorders of prothrombin are rare and involve either reduced synthesis of the Factor II molecule (referred to as hypoprothrombinemia or type I prothrombin deficiency) or the normal synthesis of a dysfunctional molecule (referred to as dysprothrombinemia or type II prothrombin deficiency). Patients with dysprothrombinemia have only 2% to 50% of the clotting activity of normal prothrombin; in these patients the severity of the bleedings correlates fairly well with the amount of prothrombin activity in plasma. A number of dysprothrombinemias have been further

characterized by amino acid sequence analysis of the isolated prothrombin molecule or by nucleotide sequence analysis of their prothrombin genes.

It is known that the gene variant Factor V Leiden (FVL-R506Q) and prothrombin G20210A (FIIG20210A) are two of the most commonly recognized genetic prothrombic risk factors for venous thrombosis. Based on the increased thrombotic tendency in venous thrombosis studies, these two gene variants have also been examined for possible association with arterial thrombosis in myocardial infarction. The prothrombin variant G20210A, comprising a G to A transition mutation at nucleotide 20210 is a very good example. This point mutation is associated with increased prothrombin levels that lead to an increased risk of thrombosis (Poortn, Blood 1996; 88 (10): 3698-703). Publications indicate an increased risk of cardiac infarctions (Rosendahl, Blood 1997; 90(5) 1747-50) and venous thromboses (Brown, Br. J. Haematol; 98(4): 907-9). However, it has also been possible to demonstrate that discrimyocardial infarctionnation between mutation carrier and the wild type is not possible with the aid of the prothrombin level, since the two groups cannot be separated (Poortn, Blood 1996; 88(10): 3698-703; see also our own results).

Several studies have shown higher prevalence of FIIG20210A in patients with myocardial infarction compared to normal controls. However, most of the results from these studies failed to achieve statistical significance, possible because of the extremely low frequency of FIIG20210A in the studied population and the use of relatively small sample sizes. Nevertheless, a few studies have presented conflicting results. Although FVL strongly correlates with deep venous thrombosis, the majority of the previous studies have failed to show a correlation of FVL to myocardial infarction. Recently, a few studies have suggested that FVL may associate with early onset myocardial infarction and myocardial infarction with normal coronary angiography.

In contrast, a common gene variant, factor XIIIV34L (FXIIIV34L) has recently been suggested to confer a protective role against myocardial infarction based on lower prevalence of FXIIIV34L in myocardial infarction patients compared with controls. However, conflicting results were also reported. Furthermore, results from function studies on the FXIIIV34L allele do not support the hypothesis of a protective role against myocardial infarction. Therefore, the role of these gene variants in the pathogenesis of myocardial infarction remains unknown.

Furthermore, no cause of myocardial infarction is detectable in a high proportion of all cases. If such defects exist, the hemostatic equilibrium is disturbed and the ratio between pro- and anticoagulatory factors is shifted in favor of one side. To this are added defects in the fibrinolysis system that reduce the breakdown of clots formed.

Being a multifactorial disorder, the genetic components of myocardial infarction may be a combined effect of a number of genes with each playing only a small role. The predisposition imparted by individual genes may act independently or interact with other genes to result in an additive effect and/or a synergistic co-effect. Common challenges facing case control studies on possible gene-gene interactions include relatively small

sample sizes, a low frequency of gene variants, and ethnic heterogeneity of the investigated population.

It is the task of the laboratory to identify such defects so that the doctor performing the treatment can estimate the individual risk to the patient and react to this. Various methods of diagnosis may be employed to identify such defects.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method for determining a risk factor for myocardial infarction. Assaying a suitable biological sample and determining the presence or absence of genetic elements that are correlated with elevated risk of myocardial infarction can perform this assessment.

In one embodiment, the invention provides a method in which said genetic elements are a mutation in the gene encoding Factor II, more particularly a G to A transition mutation at nucleotide position 20210 (FII G20210A), and a mutation in the Factor XIII gene, more particularly a valine to leucine substitution mutation at amino acid position 34 (FXIII V34L). It is to be understood that other genetic elements, coding for the components of the fibrinolysis system, the clotting system, and the complement system, can be similarly analyzed to determine a correlation with an elevated risk for myocardial infarction. In another aspect the present invention provides a kit for use in said method.

The present invention also provides a first nucleotide sequence comprising at least part of the nucleotide sequence of the human Factor II gene, wherein nucleotide G at position 20210 is replaced by A, a second nucleotide sequence comprising at least part of the nucleotide sequence of the human Factor XIII gene, wherein the DNA coding for the amino acid valine at amino acid position 34 is replaced by leucine, and a third nucleotide sequence comprising at least part of the nucleotide sequence of the human Factor V gene, wherein a residue at a position is replaced by another residue, as well as the use of such sequences for the detection of such mutations. The present invention also provides primers for the allele specific detection of these mutations of the Factor II gene at nucleotide 20210, the Factor XIII gene at amino acid 34, and the Factor V gene at its appropriate position.

BRIEF DESCRIPTION OF THE DRAWINGS

Table 1 shows the distribution of genotypes, and carrier and allele frequencies of FIIG20210A, FVL, and FXIIIV34L in MI patients and normal controls.

Table 2 shows the distribution of genotypes among MI patients with different ages of onset compared with age-matched normal controls.

Figure 1a shows a comparison of the expected and observed prevalence of combined carrier for FIIG20210A and FXIIIV34L in MI patients and normal controls.

Figure 1b shows a comparison of the expected and observed prevalence of combined carrier for FVL, and FXIIIV34L in MI patients and normal controls.

Figure 2a shows the prevalence of FXIIIV34L in MI patients and normal controls who carry the FIIG20210A allele.

Figure 2b shows the prevalence of FXIIIV34L in MI patients and normal controls who carry the FVL allele.

DETAILED DESCRIPTION

Genetic elements associated with an elevated risk of myocardial infarction may be identified by methods known in the art. One strategy to identify said genetic elements encompasses the steps of sequencing a candidate gene in a panel of probands from families with documented myocardial infarction, followed by estimating the risk factor for myocardial infarction associated with any observed sequence variation in a population based patient-control study.

The primary basis for the present invention is evidence from the inventors' case control study, simultaneously analyzing for the presence of the FVL, FIIG20210A and FXIIIV34L variants in 500 myocardial infarction patients and 500 normal individuals of a genetically isolated population of the island portion of Newfoundland and Labrador, as exemplified in Example 1. The population consists mainly of descendants of English and Irish settlers who arrived in the 18th and 19th centuries. The geographic and social isolation of the island has ensured very little inward migration for several hundred years, and thus has lead to a small population (530,000 individuals) with a relatively homogenous genetic background, ideal for the study of complex multifactorial diseases such as myocardial infarction.

Method for the detection of risk factors associated with myocardial infarction

The present invention relates to a method for the detection of a plurality of defects in a multi-stage, multi-factorial biochemical reaction system such as the fibrinolysis system, the clotting system, or the complement system, wherein the defects are associated with an increased risk of myocardial infarction in an individual, comprising the steps of screening for suitable patients at risk of myocardial infarction, obtaining a suitable biological sample from the individual, determining gene sequences or gene products of genes known to be associated with myocardial infarction, and determining the risk to the individual of myocardial infarction.

1. Screen for risk factors to select patients

It is therefore an aim to select the patient group whose risk for myocardial infarction is to be assessed before the DNA analysis is conducted. To date, these groups have been selected either according to a family history of myocardial infarction, indicative of a genetic predisposition to the condition, or on the basis of an individual case study. In a typical individual case study, a patient may either present with a prior history of myocardial infarction, or global parameters may determine the interaction of several components of the clotting system. For example, the prothrombin time (PT) is a global parameter which determines the state of the exogenous clotting system and the partial thromboplastin time (aPTT)) is a global parameter which determines the state of the endogenous clotting system. The prothrombin level can be measured in a human plasma sample or other suitable biological sample using techniques known in the art. Measuring prothrombin levels in a statistically sufficient number of healthy individuals can define a normal value.

2. *Obtaining a suitable biological sample*

If a positive family history is recorded, or a positive finding is obtained from a personal history or a global parameter such as PT or aPTT, individuals parameters can be analysed to discover the underlying genetic basis for the condition. The analysis can comprise biochemical analysis such as enzyme assay or immunoassay to determine the presence of a variant gene product, or a DNA analysis to determine the presence of a variant gene sequence. Generally, DNA analysis provides unambiguous diagnoses of these congenital defects.

The biochemical analysis comprises individual parameters, each of which determines the presence or absence of only an individual genetic product, such as variants of Factors II, V, or XIII, or any other components involved in the fibrinolysis system, the clotting system, or the complement system. For example, the presence of the gene product of the FVL allele can be determined by reaction with an antibody which specifically binds to the epitope comprising the valine to leucine substitution at amino acid position 34.

The term "specifically binds" refers to the ability of individual antibodies to specifically immunoreact with an antigen. The binding is a non-random binding reaction between an antibody molecule and an antigenic determinant of the protein. The desired binding specificity is typically determined from the reference point of the ability of the antibody to differentially bind the molecule and an unrelated antigen, and therefore distinguish between two different antigens, particularly where the two antigens have unique epitopes. An antibody that specifically binds to a particular epitope is referred to as a "specific antibody".

The DNA analysis comprises assessing individual parameter, each of which determines the presence or absence of only an individual genetic element, such as FVL, FIIG20210A or FXIIIV34L alleles, or any other genetic element involved in the fibrinolysis system, the clotting system, or the complement system. For example, the presence of the FVL allele can be determined by the use of sequence-specific oligonucleotides.

Once patients have been screened on the basis of family or case history, a suitable biological sample must be obtained in order to carry out the biochemical or DNA analysis. Biological samples obtained from a subject may contain genomic DNA, RNA, or protein. For the purposes of biochemical analysis, a suitable biological sample is preferably a blood or blood plasma sample containing the protein of interest. For the purposes of DNA analysis, a suitable biological sample may be a blood or blood plasma sample, urine, saliva, tissue biopsy, surgical specimen, fine needle aspirates, amniocentesis samples, or any other material comprising the patient's genomic DNA. Methods for obtaining suitable biological samples are known in the art.

3. Determining sequences or products of genes associated with myocardial infarction

It was hitherto unrecognized that a combined carrier status of the FIIG20210A and FXIIIV34L genetic elements is a strong risk factor for myocardial infarction. With the present invention such a correlation has now been established.

By the term "genetic element", any nucleotide sequence present in the genome of the organism under investigation is meant. Genetic elements correlated with elevated myocardial infarction risk comprise nucleotide sequences from the Factor II gene itself as well as from any other gene which might cause elevated prothrombin levels. Such genes may include, but are not restricted to, genes coding for components of the fibrinolysis system, the clotting system, or the complement system, such as, for example, the genes coding for Factor II, Factor V, Factor XIII. Aberrations in those genes may include deletions, insertions, mutations, chromosomal dislocations or other genetic mechanisms.

In Example 1, the Factor II, Factor V, and Factor XIII genes were analyzed as candidate genes in a search for genetic elements that may contribute to elevated risk of myocardial infarction. Factor II is encoded by a 21-kb-long gene localized on chromosome 11, position 11p11-q12. The prothrombin gene is organized in 14 exons, of which exon 1 comprises the 5' untranslated (UT) region and exon 14 comprises the 3'-UT region. The nucleotide sequence of the Factor II gene, its flanking sequences as well as the position of the various exons has been described previously (Biochemistry 26, 6165-6177, 1987). The G20210A sequence variation is located at the last position of the 3'-UT at or near the cleavage site in the mRNA precursor to which poly A is added. Three conserved sequences in mRNA precursors, located in the vicinity of this site, are required for cleavage and polyadenylation: the AAUAAA sequence, the nucleotide to which poly A is added, and the region downstream of this nucleotide. Generally, the nucleotide to which poly A is added is an A, mostly preceded by a C. As a consequence of the G to A transition at position 20210, a CA dinucleotide (rather than a GA dinucleotide) has been introduced at or near the cleavage and polyadenylation site.

Factor V is encoded by a gene localized on a human chromosome. The nucleotide sequence of the Factor V gene, its flanking sequences as well as the position of the various exons has been described previously. Factor XIII is encoded by a gene localized

on a human chromosome. The nucleotide sequence of the Factor XIII gene, its flanking sequences as well as the position of the various exons has been described previously.

Genetic analysis of the candidate genes comprises obtaining from the patient a biological sample, selective amplification from said sample of nucleic acid coding regions or other regulatory elements of the candidate genes comprising the genetic aberration, and analyzing the sequence for the presence of signature substitutions indicative of the gene variant of interest. Biological samples are those containing genomic DNA, cDNA, RNA, or protein obtained from the cells of a subject, such as those present in peripheral blood, urine, saliva, tissue biopsy, surgical specimen, fine needle aspirates, amniocentesis samples and autopsy material. In one example, a sample includes prostate cancer cells obtained from a subject.

Various techniques for amplifying nucleic acid are known in the art. One example of a technique for the amplification of a DNA target segment is the polymerase chain reaction (PCR). With the PCR technique the copy number of a particular target segment is increased exponentially with a number of cycles. A pair of primers is used and in each cycle a DNA primer is annealed to the 3' side of each of the two strands of the doubled stranded DNA-target sequence. The primers are extended with a DNA polymerase in the presence of the various mononucleotides to generate double stranded DNA. The strands of the double stranded DNA are separated from each other by thermal denaturation and each strand then serves as a template for primer annealing and subsequent elongation in a following cycle. The PCR method has been described in Saiki et al., Science 239, 487, 1988 and in European Patents no. EP 200 362 and EP 201 184. Other amplification techniques include mismatch PCR, the Ligase Chain Reaction (LCR) and the Repair Chain Reaction (RCR).

The term "oligonucleotide" as used herein refers to a linear polynucleotide molecule of up to about 200 nucleotide bases in length, for example a polynucleotide (such as DNA or RNA) which is at least about 6 nucleotides, for example at least 15, 50, 100 or 200 nucleotides long. These oligonucleotides may function as primers and probes.

The term "primer" as used herein refers to an oligonucleotide either naturally occurring (e.g. as a restriction fragment) or produced synthetically, which may act as a point of initiation of synthesis of a primer extension product and which is able to hybridize to a nucleic acid strand (template or target sequence) when placed under suitable conditions (e.g. buffer, salt temperature and pH) in the presence of nucleotides and an agent for nucleic acid polymerization, such as DNA dependent or RNA dependent polymerase. A primer must be sufficiently long to prime the synthesis of extension products in the presence of an agent for polymerization. A typical primer contains at least about 10 nucleotides in length of a sequence substantially complementary or homologous to the target sequence, but somewhat longer primers are preferred. Usually primers contain about 15-26 nucleotides, but longer primers may also be employed. Normally a set of primers will consist of at least two primers, one `upstream` and one `downstream` primer which together define the sequence that will be amplified using said primers. The primers used for amplification of the various regions of the target genes may be synthesized by

the methoxyphosphoramite method (Tetrahydron Letters 22, 1859-1865, 1981) or other suitable method known in the art. The sequences of the primers are chosen such that they flank the aberrant regions of the target gene. Suitable primers for amplification of the various transcribed and untranscribed regions of the alleles of interest are those determined to be most efficacious for the task.

Detection of mutations may, inter alia, be performed by methods known in the art, such as direct sequence analysis of amplified nucleic acid containing the aberration, by allele specific amplification which differentially amplifies DNA containing and DNA lacking the aberration, or by restriction fragment analysis. It may also be performed by hybridization with a probe that is able to differentially hybridize under stringent conditions to the stretch of amplified nucleic acid material which may contain the aberration.

Stringent (hybridization) conditions are conditions under which a test nucleic acid molecule will hybridize to a target reference nucleotide sequence, to a detectably greater degree than other sequences (e.g., at least two-fold over background). Stringent conditions are sequence-dependent and will differ in experimental contexts. For example, longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5.degree. C. to about 20.degree. C. lower, and preferably, 5.degree. C. lower, than the thermal melting point (Tm) for the specific target sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion concentration (or other salts), typically about 0.01 to 1.0 M Na ion concentration (or other salts), at pH 7.0 to 8.3, and the temperature is at least about 30.degree. C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60.degree. C. for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37.degree. C., and a wash in 2.times.SSC at 50.degree. C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37.degree. C., and a wash in 0.1.times.SSC at 60.degree. C.

The term "probe" refers to an oligonucleotide, typically labeled, that forms a duplex structure with a sequence of a target nucleic acid due to complementary base pairing. The probe will comprise a hybridizing region, preferably consisting of 10 to 50 nucleotides, more preferably 20 to 30 nucleotides, corresponding to a region of the target sequence. The hybridizing region of a probe is preferably identical or fully complementary to the sequence of the target region. The hybridizing region may also contain a certain number of mismatches, those skilled in the art of nucleic acid technology can determine duplex stability considering a number of variables including the length and base-pair composition of the probe, ionic strength of the buffer, reaction temperature and incidence of mismatched base pairs, see, e.g. Sambrook et al., Molecular cloning: A laboratory manual, second edition (1989) Cold Spring Harbor Laboratory Press.

The term "label" as used herein refers to any atom or molecule which can be attached to a nucleic acid and which can be used either to provide a detectable signal or to interact with a second molecule to modify the detectable signal provided by said second molecule. Preferred labels are radioisotopes, fluorescent compounds, enzymes or chemoluminescent compounds.

Hybridization of the probe with the target sequence may be detected by techniques known in the art of nucleic acid technology such as Northern or Southern blotting, see e.g. Sambrook et al., supra. Preferred analysis systems are electrochemical infarction luminescence (ECL) based analysis or enzyme linked gel assay (ELGA) based analysis.

Sequence analysis includes direct analysis of the DNA sequence flanking and constituting the exons and untranslated regions of the prothrombin gene. This method involves any protocol that is currently available to any person skilled in the art for directly determining DNA- or RNA sequences, such as the dideoxynucleotide method described by Sanger (Proc. Natl. Acad. Sci. USA, 74 5463-7, 1977).

It is also possible to analyze the amplified material through restriction fragment analysis. In this method the amplified material is digested with restriction enzymes that recognize DNA sequences that are either present in DNA sequences derived from patients carrying an aberration in the exons or untranslated regions of the prothrombin gene, or that are present in the native sequence encoding prothrombin.

It is also possible to analyze a known mutation by allele specific amplification (Trends in Genetics, 12, 391-392, 1996 and Mullis et al. eds, The polymerase chain reaction, Birkhauser, Boston, Basel, Berlin, 1994, pp1-13) Allele specific PCR for the FIIG20210A variation has been described in Thrombosis and Haemostasis 78, 1157-1163 (1997). This technique is based on the observation that under certain conditions primer elongation cannot take place when the 3' terminal nucleotide of a primer is not complementary to the template. With the use of two forward primers that differ only at their terminal 3' nucleotide it is possible to distinguish between homozygous or heterozygous individuals, for instance with respect to their FIIG20210A alleles: analysis of material from homozygous individuals will result in a positive amplification result with either one of the primers, material from heterozygous individuals will result in a positive amplification with both primers. Similar analysis can be performed for the FVL, and FXIIIV34L alleles, as well as for alleles of any other genes involved in the fibrinolysis system, the clotting system, or the complement system.

Biochemical analysis of the products of candidate alleles comprises immunological or physiochemical detection.

Immunological detection comprises the steps of obtaining a sample of the patient's blood, plasma, or other bodily fluid in which the gene product may or may not be present, adding a known quantity of an antibody specific to the variant gene product under condition which favor binding of the specific antibody to the variant gene product, and

assessing the presence or absence of the binding of the antibody to the variant gene product. A positive test result wherein such binding is observed is indicative of the presence of the gene product, and a negative test result wherein such binding is not observed is indicative of the absence of the gene product.

For the purpose of this application, an antibody is an immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen.

A naturally occurring antibody (e.g., IgG) includes four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. However, the antigen-binding function of an antibody can be performed by fragments of a naturally occurring antibody. Thus, these antigen-binding fragments are also intended to be designated by the term antibody. Examples of binding fragments encompassed within the term antibody include (i) an Fab fragment consisting of the VL, VH, CL and CH1 domains; (ii) an Fd fragment consisting of the VH and CH1 domains; (iii) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (iv) a dAb fragment (Ward *et al.*, Nature 341:544-6, 1989) which consists of a VH domain; (v) an isolated complimentarity determining region (CDR); and (vi) an F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region. Furthermore, although the two domains of the Fv fragment are coded for by separate genes, a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain Fv (scFv); Bird *et al.* Science 242:423-6, 1988; and Huston et al., *Proc. Natl. Acad. Sci.* 85:5879-83, 1988) by recombinant methods. Such single chain antibodies are also included.

In one example, antibody fragments are capable of crosslinking their target antigen, e.g., bivalent fragments such as F(ab')2 fragments. Alternatively, an antibody fragment which does not itself crosslink its target antigen (e.g., a Fab fragment) can be used in conjunction with a secondary antibody which serves to crosslink the antibody fragment, thereby crosslinking the target antigen. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described for whole antibodies. An antibody is further intended to include bispecific and chimeric molecules that specifically bind the target antigen.

"Specifically binds" refers to the ability of individual antibodies to specifically immunoreact with an antigen. The binding is a non-random binding reaction between an antibody molecule and an antigenic determinant of the T cell surface molecule. The desired binding specificity is typically determined from the reference point of the ability of the antibody to differentially bind the T cell surface molecule and an unrelated antigen, and therefore distinguish between two different antigens, particularly where the two antigens have unique epitopes. An antibody that specifically binds to a particular epitope is referred to as a "specific antibody".

Physiochemical detection comprises the steps of obtaining a sample of the patient's blood, plasma, or other bodily fluid in which the gene product may or may not be present,

optionally isolating the gene product from the sample by immunological or physical means, and assessing the presence or absence of the variant gene product.

For the purpose of this publication, an "isolated" biological component (such as a nucleic acid molecule or protein) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs (i.e. other chromosomal and extrachromosomal DNA and RNA). Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids and proteins. The term "isolated" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a substantially purified protein or nucleic acid preparation is one in which the protein or nucleic acid referred to is more pure than the protein in its natural environment within a cell or within a production reaction chamber (as appropriate). For example, a preparation of a modified protein is purified if the protein represents at least 50%, for example at least 70%, of the total protein content of the preparation. Methods for purification of proteins and nucleic acids are well known in the art. Examples of methods that can be used to purify a protein include, but are not limited to the methods disclosed in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Ch. 17).

By "isolating the gene product from the sample by immunological means" is meant, for example, that the variant gene product may be isolated from the patient sample by means of an antibody capable of detecting both the wild-type and the variant form of the gene product (that is, an antibody directed to an epitope conserved in both the wild-type and the variant gene product, but unique to the products of that gene locus). By "isolating the gene product from the sample by physical means" is meant, that the variant gene product may be isolated from the patient sample by means of centrifugation, chromatography, electrophoresis, or other techniques known toi the worker skilled in the art.

By "physiochemically assessing the presence or absence of the variant gene product" is meant the use of techniques such as protein kinetics, spectroscopy, crystallography or other techniques known to workers skilled in the art to distinguish the unique physiochemical signature of the variant gene product from that of the native or wild-type protein.

4. Determining the risk of myocardial infarction to the individual.

As is illustrated in example 1, the G20210A mutation in the Factor II gene, combined with the V34L mutation in the Factor XIII gene, has been demonstrated to be present in a group of patients exhibiting a risk for myocardial infarction without the cause thereof having been previously determined. Example 1, which illustrates the general use of the methods for the detection of mutations indicative of an elevated risk for myocardial infarction, is provided for illustrative purposes only.

The study in example 1 shows that the presence of sequence variation G20210A in the Factor II gene, combined with the sequence variation V34L in the Factor XIII gene, is a risk factor for myocardial infarction (odds ratio: 2.8; 95% confidence interval: 1.4 to 5.6; Table 1). This study does not show the mechanism by which the G20210A allele of the Factor II gene may interact with the V34L allele of the Factor XIII gene to contribute to elevated myocardial infarction risk.

Once the genetic material from the patient's sample has been analyzed for the presence of the sequence variation in the target gene, or the biological material from the patient's sample has been analyzed for the presence of the variant product of the target gene, the risk for myocardial infarction can be determined on the basis of the risk established in a case-control study such as, for example, the Newfoundland study of Example 1.

Kit for the detection of risk factors associated with myocardial infarction

Kit for the detection of risk factors associated with myocardial infarction may be kits for the genetic, immunological or physiochemical detection of risk factors associated with myocardial infarction.

Kit for the genetic detection of risk factors associated with myocardial infarction comprise oligonucleotides specific to the variant region of the alleles of interest or to sequence flanking the variant region, buffers, nucleotides, enzymes such as polymerases, ligases or endonucleases as appropriate to the specific method of genetic analysis known in the art, and other reagent useful in performing such analysis.

Kit for the immunological detection of risk factors associated with myocardial infarction comprise primary antibodies (monoclonal, polyclonal or purified) specific to the variant epitopes of the gene products of interest, buffers, membranes, secondary antibodies, and preferably labeled secondary antibodies specific to the primary antibodies, and other reagent useful in performing such analysis

Kit for the physiochemical detection of risk factors associated with myocardial infarction comprise electrophoresis buffers, centrifugation buffers, substrates specific to the variant gene products, and preferably chromogenic substrates, and other reagent useful in performing such analysis.

EXAMPLES

Example 1

SUBJECTS, MATERIALS AND METHODS

Subjects:

Blood samples were collected from 500 consecutive myocardial infarction patients and 500 normal controls of the genetically isolated Newfoundland population. Patients categorized in the myocardial infarction group represented those presenting to the emergency department or within one of the Health Care Corporation of St. John's hospitals with symptoms and biochemical evidence suggestive of myocardial infarction. Only patients with cardiac Troponin I values greater than 2.0 μg/L (Axsym, Abbott Diagnostics) or greater than 0.5 μg/L (Access II, Beckman-Coulter Corp.) were used in this group. Control subjects were selected from consecutive individuals without prior history of myocardial infarction or thrombosis presenting to the emergency department for trauma, accidental injury, or other non-cardiac and non-thrombotic related events. Discarded blood samples collected for complete blood count were used for DNA extraction and analysis. Ethics approval for this study was granted by the Human Investigations Committee of Memorial University and by the Health Care Corporation of St. John's.

Genotyping of FIIG20210A, FVL, and FXIIIV34L:
Genomic DNA was isolated from the peripheral blood using standard methods (a:35-36). Genotyping of the FVL, FIIG20210A, and FXIIIV34L was performed by PCR amplification of each of the target alleles from genomic DNA followed by restriction digestion with each of corresponding enzymes ***MnlI*, *HindIII*** and ***DdeI*** respectively, as previously described (a:36). The digested PCR products were separated by electrophoresis in 10% polyacrylamide gels and visualized by staining with ethidium bromide.

Prevalence determination and association study:
The prevalence of each gene variant was calculated by counting the total carrier frequency including heterozygotes and homozygotes. The allele frequencies were determined by gene counting. Pearson Chi Square statistical analysis was performed using SPSS v10.0 to test the association between genotypes and the prevalence of myocardial infarction. Odds ratios (OR) were calculated as a measure of the relative risk for myocardial infarction and were given with 95% confidence intervals.

Analysis of gene-gene interaction:
Gene-gene interactions were determined, first by comparing the prevalence of combined carrier for two of the three gene variants in patients and controls; and second by analysis of the distribution of one chosen gene variant in sub-grouped patients and controls who carry another gene variant as genetic background.

RESULTS

Genotyping FIIG20210A, FVL, and FXIIIV34L:
The genotype distributions, carrier frequencies, and allele frequencies of FIIG20210A, FVL, and FXIIIV34L in both the myocardial infarction patient and the control populations are given in Table 1. The FIIG20210A allele was detected in 3.2% of patients which was significantly higher than the 1 % observed in controls (OR 3.3, 95% CI 2.6-4.0; P=0.015). An identical prevalence of FVL was observed in both patient and control

populations (4.6% vs. 4.6%). No homozygotes for either FIIG20210A or FVL allele were found in either population. In both patient and control populations, FXIIIV34L had similar prevalence (47.0% vs. 47.8%) and allele frequency (27.7% vs. 27.1 %). The prevalence of homozygous V34L was higher in patients compared with controls (8.4% vs. 6.4%), but the difference did not reach statistical significance. It is to be understood that, because the statistical significance of the prevalence of a genetic element as it relates to myocardial infarction risk is dependent on the sample size, an increase in the sample size will conclusively determine said statistical significance.

The distribution of the three gene variations was further analyzed by sub-grouping patients and controls according to age, as shown in Table 2. Myocardial infarction patients were divided into those with an early age of onset (less than 50 years) and those with a later age of onset (greater than 50 years). The control population was also divided into the two corresponding age groups. Interestingly, a disequilibrium distribution of the FVL allele was observed in the early onset patient group. The FVL allele was detected in 13.0% of patients with early age of onset, which was significantly higher than the 3.8% in patients with a later age of onset (OR: 3.9, 95 % CI 3.3-4.4; P=0.004) and the 4.8 % in the age matched controls (OR: 3.0, 95 % CI 2.2-3.7; P=0.007). The prevalence of FIIG20210A was also significantly higher in the early onset group of myocardial infarction (4.3%) compared to the controls under age 50 (0.8%). Although, the prevalence of FIIG20210A was slightly higher in the early onset group compared to the later onset group, this difference did not achieve statistical significance. The FXIIV34L showed a slight but statistically insignificant difference in prevalence between the patients with early and later onset age.

Gene-gene Interactions:
Interaction between FXIIIV34L and FIIG20210A was first analyzed by comparing the prevalence of combined carriers (individuals carrying both FIIG20210A and FXIIIV34L) in the total patient and control populations with their corresponding theoretical prevalence of combined carriers. Using carrier frequencies described in Table 1, the theoretical prevalence for combined carriers of FIIG20210A and FXIIIV34L is 1.5% (47.0% x 3.2%) in myocardial infarction patients and 0.48% (47.8% x 1%) in controls (Figure la). The observed prevalence of combined carriers in the myocardial infarction patient population (2.4%; 12 of 500) was 1.6-fold higher than its theoretic prevalence (1.5%), and in normal control population (0.2%; 1 of 500) was 2.4-fold lower than its theoretic expected prevalence (0.48%). The observed prevalence of combined carriers was 12-fold higher in myocardial infarction patient compared with the control population (P = 0.002).

The interaction between the FIIG20210A and the FXIIIV34L was further examined by analysis of the distribution of FXIIIV34L in sub-grouped patients and controls who carry FIIG20210A as a genetic background. Although the FXIIIV34L showed an almost equal distribution in our myocardial infarction patient and control populations, FXIIIV34L alleles were detected in 75.0% (12 of 16) patients with a genetic background of FIIG20210A but only in 20.0% (1 of 5) of controls with the same genetic background (OR 3.7. 95 % CI 2.4-5.1; P=0.013) (Figure 2).

Of 13 combined carriers of FIIG20210A and FXIIIV34L identified from the studied population (500 patients and 500 controls), 12 subjects (92.3 %) belonged to the myocardial infarction patient population but only 1 (0.7%) from the control population. The co-existence of these two gene variants imparts a strong predisposition for myocardial infarction with high penetrance.

The prevalence of combined carriers of FVL and FXIII34L were similar in both myocardial infarction patient (12 of 500, 2.4%) and control (9 of 500, 1.8%) groups and was consistent with their expected frequencies (2.16% in patients and 2.2% in controls; Figure lb). We further analyzed the prevalence of FXIIIV34L in patients and controls who carry the FVL allele as a genetic background. The prevalence of FXIIIV34L was slightly higher in the sub-grouped controls (13 of 23, 56.52%) than in the sub-grouped patients (9 of 23. 39.1%) but the difference was not statistically significant.

There were no combined carriers of FIIG20210A and FVL in the myocardial infarction patient or control populations. This is expected considering a calculated expected frequency of combined carriers of 0.13 % in myocardial infarction patients and 0.01 % in normal controls, respectively.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for the detection of defects in a multi-stage, multi-factorial biochemical reaction system, wherein the defects are associated with an increased risk of myocardial infarction in an individual, comprising the steps of:
   a) screening for suitable patients at risk of myocardial infarction, wherein said screening is conducted on the basis of a family history or individual case history;
   b) obtaining a suitable biological sample from the individual;
   c) determining the presence of variant gene products or sequences of genes known to be associated with myocardial infarction; and
   d) determining the risk to the individual of myocardial infarction.

2. The method as in claim 1, wherein the multistage, multifactorial biochemical reaction system is selected from the group comprising the fibrinolysis system, the clotting system, and the complement system.

3. The method as in claim 1, wherein the biological sample is a blood or blood plasma sample, urine, saliva, tissue biopsy, surgical specimen, fine needle aspirates, amniocentesis samples, or any other material comprising the patient's genomic DNA.

4. A method for determining whether an individual is at an increased risk for myocardial infarction, comprising detecting the presence or absence of mutations that are correlated with an elevated risk for myocardial infarction.

5. A method for determining whether an individual is at an increased risk for myocardial infarction, comprising detecting the presence or absence of aberrant gene products of genes that are correlated with an elevated risk for myocardial infarction.

6. The method as in claim 5, wherein genes known to be associated with myocardial infarction are at least two genes selected from the group comprising the genes encoding Factor II, Factor V, and Factor XIII.

7. The method as in claim 6, wherein the sequence of the Factor II gene comprises SEQ ID NO:1.

8. The method as in claim 6, wherein the sequence of the Factor V gene comprises SEQ ID NO:2.

9. The method as in claim 6, wherein the sequence of the Factor XIII gene comprises SEQ ID NO:3.

10. The method as in claim 6, wherein the presence of at least two of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, is indicative of an increased risk for myocardial infarction in said individual.

11. The method as in claim 6, wherein the presence of at least two of FIIG20210A, FVL and FXIIIV34L gene products is indicative of an increased risk for myocardial infarction in said individual.

12. A method for determining whether an individual is at an increased risk for myocardial infarction, comprising determining the Factor II and Factor XIII gene sequences of an individual, whereby the presence of a G20210A mutation in the Factor II gene sequence, and the presence of a V34L mutation in the Factor XIII gene sequence is indicative of an increased risk for myocardial infarction in said individual.

13. A method for determining whether an individual is at an increased risk for myocardial infarction, comprising determining the Factor II and Factor XIII gene products of an individual, whereby the presence of the FIIG20210A and the FXIIIV34L gene products is indicative of an increased risk for myocardial infarction in said individual.

14. The method as in any of claim 1 to 13, wherein the method is an in vitro method.

15. The method as in claim 12, wherein the presence or absence of the genetic mutations is determined by performing a nucleic acid amplification reaction.

16. The method as in claim 12, wherein the nucleic acid amplification reaction is an allele specific polymerase chain reaction.

17. The method as in claim 13, wherein the presence of the FIIG20210A and the FXIIIV34L gene products is determined a method selected from the group comprising immunoassay, enzyme assay, centrifugation, crystallization, electrophoresis and spectroscopy.

18. A kit for determining by means of DNA analysis whether an individual is at an increased risk for myocardial infarction, comprising oligonucleotides specific to the variant region of the alleles of interest or to sequence flanking the variant region, buffers, nucleotides, enzymes such as polymerases, ligases or endonucleases as appropriate to the specific method of genetic analysis known in the art, and other reagent useful in performing such analysis.

19. A kit for immunologically determining whether an individual is at an increased risk for myocardial infarction, comprising primary antibodies (monoclonal, polyclonal or purified) specific to the variant epitopes of the gene products of interest, buffers, membranes, secondary antibodies, and preferably labeled

secondary antibodies specific to the primary antibodies, and other reagent useful in performing such analysis.

20. A kit for physiochemically determining whether an individual is at an increased risk for myocardial infarction, comprising electrophoresis buffers, centrifugation buffers, crystallization buffers, substrates specific to the variant gene products, and preferably chromogenic substrates, and other reagent useful in performing such analysis.

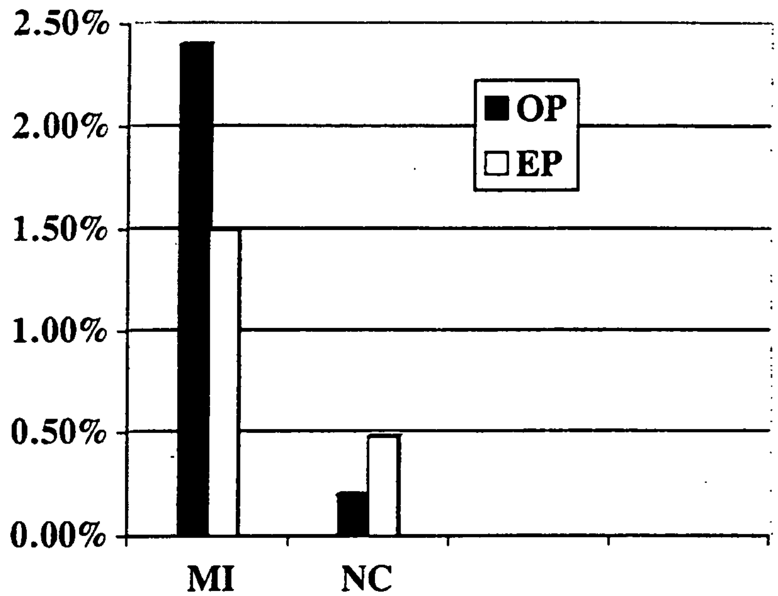
Tab-1: Distributions of genotypes, and carrier and allele frequencies of FIIG20210A, FVL and FXIIIV34L in MI patient and normal control (NC) populations.

	Genotype	MI (n = 500)	NC (n = 500)	OR	P value
	G/G	484 (96.8%)	495 (99.0%)		
FIIG20210A	G/A	16 (3.2%)	5 (1.0%)		
	A/A	0 (0%)	0 (0%)		
Carrier F.		3.2%	1.0%	3.3	0.015
Allele F.		1.6%	0.5%		
	R/R	477 (95.4%)	477 (95.4%)		
FVL(R506Q)	R/Q	23 (4.6%)	23 (4.6%)		
	Q/Q	0 (0%)	0 (0%)		
Carrier F.		4.6%	4.6%	1.00	NS
Allele F.		2.3%	2.3%		
	V/V	265 (53.0%)	261 (52.2%)		
FXIIIV34L	V/L	193 (38.6%)	207 (41.4%)		
	L/L	42 (8.4%)	32 (6.4%)		
Carrier F.		47.0%	47.8%	0.97	NS
Allele F.		27.7%	27.1%		

Table 2: Distribution of genotypes among MI patients with different onset ages and compared with age-matched normal controls (NC).

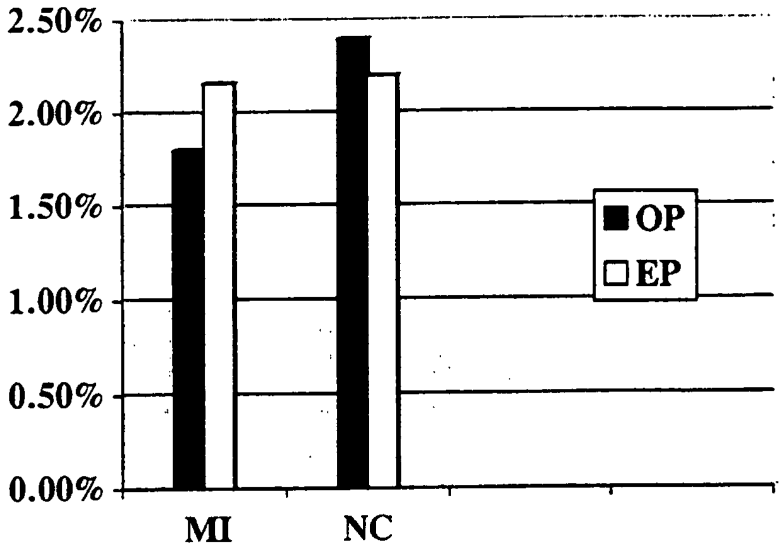
	MI	NC	OR	P
FII 20210A Carriers	**16/500 (3.2%)**	**5/500 (1%)**	**3.3**	**0.015**
Age ≤ 50Y	2/46 (4.3%)	3/373 (0.8%)	5.6	0.04
Age > 50Y	14/454 (3.1%)	2/127 (1.6%)	2.0	NS
FVL Carriers	**23/500 (4.6%)**	**23/500 (4.6%)**	1.0	NS
Age ≤ 50Y	6/46 (13.0%)	18/373 (4.8%)	3.0	0.007
Age > 50Y	17/454 (3.8%)	5/127 (3.9%)	1.0	NS
FXIII 34LCarriers	**235/500 (47.0%)**	**239/500 (47.8%)**	**1.0**	NS
Age ≤ 50Y	19/46 (41.3%)	176/373 (47.2%)	0.8	NS
Age > 50Y	216/454 (47.6%)	63/127 (49.6%)	0.9	NS

Fig-1a: Comparison of the expected and observed prevalence of combined carrier for FII20210A and FXIII V34L among MI patients and controls.



OP: Observed prevalence; EP: expected prevalence (prevalence of FIIG20210A x prevalence of FXIII); Combined carriers in MI = 12 out of 500; Combined carriers in MI = 1 out of 500

Fig-1b: Comparison of the expected and observed prevalence of combined carrier for FVL and FXIII V34L among MI patients and controls.



OF: Observed prevalence: EF: expected prevalence: Combined carriers in MI = 9 out 500

Combined carriers in MI = 12 out 500

Figure-2a: Prevalence of FXIII34L in MI patients and normal controls (NC) who carry the FII20210A allele.

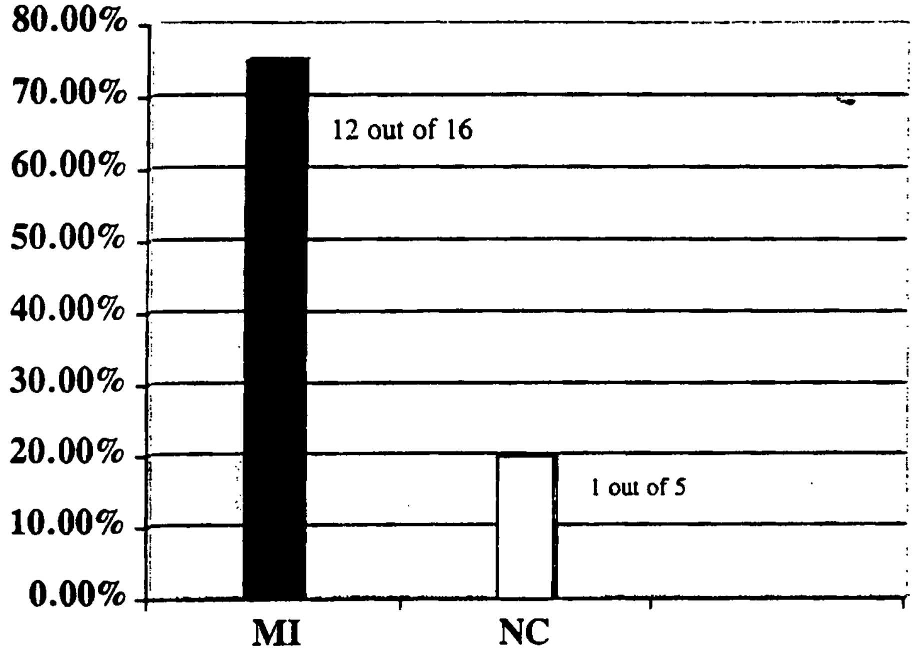


Figure-2b: Prevalence of FXIII34L in MI patients and normal controls (NC) who carry the FVL allele.